Activated T Cells to Die via Fas


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Cutting Edge: Rac GTPases Sensitize Activated T Cells to Die via Fas

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In activated CD4+ T cells, TCR restimulation triggers apoptosis that depends on interactions between the death receptor Fas and its ligand, FasL. This process, termed restimulation-induced cell death (RICD), is a mechanism of peripheral immune tolerance. TCR signaling sensitizes activated T cells to Fas-mediated apoptosis, but what pathways mediate this process are not known. In this study we identify the Rho GTPases Rac1 and Rac2 as essential components in restimulation-induced cell death. RNA interference-mediated knockdown of Rac GTPases greatly reduced Fas-dependent, TCR-induced apoptosis. The ability of Rac1 to sensitize T cells to Fas-induced apoptosis correlated with Rac-mediated cytoskeletal reorganization, dephosphorylation of the ERM (ezrin/radixin/moesin) family of cytoskeletal linker proteins, and the translocation of Fas to lipid raft microdomains. In primary activated CD4+ T cells, Rac1 and Rac2 were independently required for maximal TCR-induced apoptosis. Activating Rac signaling may be a novel way to sensitize chronically stimulated lymphocytes to Fas-induced apoptosis, an important goal in the treatment of autoimmune diseases. The Journal of Immunology, 2007, 179: 6384–6388.

Antibodies and reagents

Abs were obtained from Kamiya (Apo1-1), Alexis Biochemicals (Apo1-3), eBioscience (anti-CD3; clone OKT3), Upstate Biotechnology (Rac1), CellSignaling Technology (ERK/p-ERK, JNK/p-JNK, p-ERM, where p stands for “phosphorylated”), and Lab Vision (ezrin). Rac1-validated stealth RNA duplex (5'-UUGGAGAUAUAUCUCCUUGUCUUU-3’) consisting of a 25-mer synthetic small interfering RNA (siRNA) duplex and Rac2 siRNA were from Invitrogen. The Rac1 and Rac2 short hairpin RNA constructs in pCM5-1-H1p-EGFP vectors have been described previously (10). The Rac dominant negative construct was a gift from S. Shaw (NCI) and the FasL construct was from J. Tschopp (University of Lausanne, Lausanne Switzerland). The constitutively active Rac1 mutants were obtained from D. Cantrell (University of Dundee, Dundee, Scotland).

Real-time quantitative RT-PCR analysis

mRNA was reverse transcribed and amplified using the SuperScript One-Step RT-PCR kit with the addition of a 1/50 dilution of ROX reference dye. Primer
and probe sets for human Fasl, Rac1, Rac2, and β2-microglobulin were obtained from Applied Biosystems. Relative mRNA levels were calculated after normalizing for β2-microglobulin and control threshold cycle (Ct) values.

Quantitation of ezrin/radixin-moesin (ERM) phosphorylation
Jurkat cells were surface stained with anti-CD3 biotin for 30 min on ice and cross-linked with streptavidin at 37°C for the indicated periods of time. p-ERM intracellular staining was performed as described previously (11).

Cell death assays
Cell death assays were performed on Jurkat cells or primary T cells activated for 48 h with Con A or anti-CD3/28 and expanded in IL-2 for a minimum of 4 days. Cells were restimulated with anti-CD3 for 6–8 h, after which cells were stained with annexin/PI for 20 min and analyzed by FACS. Cell death was calculated using the formula [(1-percentage of live treated cells)/(percentage of live untreated cells)], where cells that remained Annexin- and PI-negative were considered live cells.

Lipid raft analysis
siRNA-transfected Jurkat cells (50 × 10⁶) were treated or left unstimulated with 500 ng/ml anti-CD3 for 90 min and cell membranes were fractionated as described previously (11).

Results and Discussion
TCR-induced sensitization to Fas-induced apoptosis depends on Rac GTPases
The Jurkat T lymphoma cell line is classified as type II in that Fas-induced apoptosis is dependent on mitochondrial amplification. Only cross-linked anti-Fas (Apo1–3 (IgG3) plus anti-IgG3), but not bivalent anti-Fas (Apo-1 (IgG1)) Abs induce efficient apoptosis (8, 12). Stimulation of Jurkat cells with soluble anti-CD3 synergistically induced apoptosis mediated by Apo1–1, which was similar to our previous results in primary activated human T cells (8) (Fig. 1A). A Rac1 dominant negative construct suppressed TCR sensitization to Fas-induced apoptosis, whereas inhibition of PI3K or NF-κB signaling had no effect (data not shown). Transfection of Jurkat cells with siRNA targeting endogenous Rac1 resulted in almost complete abrogation of TCR-mediated sensitization to Fas (Fig. 1B), whereas cell death induced by cross-linked anti-Fas was unaffected (Fig. 1C). This siRNA oligomer efficiently reduced Rac1 mRNA and protein but not mRNA for Rac2, a highly homologous protein present in immune cells (Fig. 1, D and E). Control and Rac1 siRNA transfection did not affect growth rates and surface levels of Fas and TCR (data not shown). Similar results were obtained with short hairpin RNA constructs targeting different sequences in Rac1 (data not shown). These data indicate that Rac1 is essential for TCR-induced apoptosis through Fas in Jurkat cells.

The cytoskeletal remodeling activity of Rac1 mediates TCR-induced “competency to die” via Fas
In resting T cells, TCR-induced changes in the dynamics of the actin cytoskeleton are important during TCR-induced activation in forming and maintaining the T cell/APC immune synapse and the subsequent proliferation and activation of T cell effector functions. Rac1 mediates this process through the dephosphorylation of ERM family linker proteins, which allows redistribution of ERM-linked surface receptors and morphological changes (11). We found that TCR stimulation of Jurkat cells induces rapid ERM dephosphorylation, which was inhibited by Rac1 siRNA (Fig. 2, A and B). Rac GTPases are activated upon TCR stimulation by the Vav1 GTP/GDP exchange factor. However, Vav-dependent events such as calcium flux, activation of JNK and ERK kinases, and NF-κB activity were not affected by Rac1 siRNA (data not shown), indicating that Rac1 controls a more restricted set of signaling pathways than Vav1.

To determine which functions of Rac1 are sufficient to mediate TCR-induced Fas sensitization, we studied J.Vav1 cells, which are unable to activate Rac1 after TCR stimulation (13). These cells are completely resistant to TCR-induced sensitization to Apo1-1, but still undergo apoptosis in response to cross-linked anti-Fas (data not shown). Transfection of RacQ61L, a constitutively active mutant of Rac1, restored the ability of TCR stimulation to sensitize to Apo1-1-induced apoptosis in J.Vav1 cells (Fig. 2C). Transfection with RacQ61L/Y40C, a mutant that is deficient in the activation of MAP kinases but not in the induction of cytoskeletal changes (14), also rescued “competency to die.” However, cells transfected with the RacQ61L/F37A mutant that is defective in modifying the cytoskeleton exhibited reduced and statistically insignificant TCR-induced sensitization to Fas (Fig. 2C). These data suggest that Rac1 effector functions that activate cytoskeletal remodeling are necessary to sensitize cells to Fas-induced apoptosis. However, the fact that TCR signaling is still necessary to sensitize cells to Fas-induced apoptosis, even in the presence of a dominant active Rac1, indicates that other Vav1-independent signals are also required.
TCR-induced apoptosis depends on both the sensitization of Fas signaling and de novo FasL synthesis. Unlike sensitization signals induced by weak TCR stimuli such as soluble anti-CD3, cross-linked anti-CD3 leads to the induction of FasL, which can then trigger apoptosis. As shown in Fig. 3A, Rac1 siRNA potently blocked the ability of Jurkat cells to undergo apoptosis in response to cross-linked anti-CD3. Surprisingly, Rac1 siRNA also reduced the transcriptional up-regulation of FasL via anti-CD3 (Fig. 3B). To determine whether this reduction in FasL production was biologically significant, we tested the ability of Rac1 siRNA-treated Jurkat cells to kill the Fas-sensitive B cell line SKW 6.4 after stimulation with bead-bound anti-CD3. Rac1 siRNA significantly reduced target cell apoptosis at two different effector-target ratios (Fig. 3C). To determine whether FasL production is limiting or whether Rac1 delivers an independent Fas sensitization signal, we labeled control siRNA-treated Jurkat cells with CFSE and mixed them at a 1:1 ratio with unlabeled Rac1 siRNA-treated cells. These cell mixtures were then stimulated with anti-CD3 and assessed for apoptosis. As shown in Fig. 3D, the addition of Rac1-sufficient cells could not rescue the apoptosis defect in the Rac1 siRNA-treated cells.

We also tested whether the transfection of FasL could restore apoptosis in Rac1-depleted Jurkat cells. As previously reported (8), transfection of FasL did not induce apoptosis alone, but as little as 10 ng/ml soluble anti-CD3 sensitized FasL-transfected cells to die. Rac1 depletion via siRNA significantly reduced the ability of TCR stimulation to sensitize FasL-transfected cells to die (Fig. 3E). These results indicate that Rac1 is required in a cell-autonomous fashion to transduce the TCR “competency to die” signal independently of its effect on FasL synthesis. Importantly, these experiments also show that Rac1 is involved in sensitizing T cells to apoptosis induced by endogenously synthesized FasL.

We have previously shown that TCR-induced sensitization to Fas requires Fas translocation to the detergent-insoluble lipid raft fraction of the membrane (8). We therefore asked whether...
Rac1 siRNA suppresses lipid raft translocation of Fas. In control Jurkat cells, TCR stimulation increased the fraction of Fas in lipid rafts 5-fold (Fig. 3F, top panel, from 6.68 to 34.32%). Rac1 siRNA-transfected Jurkat cells exhibited dramatic inhibition of Fas translocation to lipid rafts (Fig. 3F, lower panel, from 7.28 to 0.39%), indicating that Rac1 is responsible for this key step in the acquisition of sensitivity to die via Fas.

To investigate the role of Rac GTPases in the apoptosis of primary T cells, we introduced Rac1 siRNA into activated human CD4+ T cells via nucleofection. As seen in Fig. 4, A and B, Rac1 siRNA suppressed TCR-mediated sensitization to bivalent anti-Fas-induced apoptosis but did not affect the responses to cross-linked anti-Fas (data not shown). Rac1 siRNA was also efficient in reducing endogenous Rac1 protein levels in these cells (Fig. 4B, Inset). When Rac2 siRNA or a mixture of Rac1 and Rac2 siRNA was used, we found significant suppression of anti-CD3-induced death (Fig. 4C). Thus both Rac1 and Rac2, with the predominant contribution of Rac2, are likely involved in TCR-induced apoptosis of primary T cells. CD44, a surface glycoprotein that is highly expressed on activated T cells, is a hyaluronan receptor and participates in cell adhesion and motility through interaction with and modification of the extracellular matrix (15). CD44 is known to induce cytoskeletal changes through the activation of Rac1 (16). Strikingly, the treatment of human activated T cells with anti-CD44 sensitized cells to Fas-induced apoptosis as efficiently as anti-CD3 (Fig. 4D).

To extend these studies to cells genetically deficient in Rac GTPases, we examined activated mouse T cells that have been engineered to lack Rac1 or Rac2 (C. Dumont, M. Walmsley, and V. L. Tybulewicz, unpublished data). Rac2 knockout (Rac2−/−) mice are viable and have normal T cell development (17). T cell specific Rac1 knockout mice (Rac1−/−) were generated by crossing previously described mice homozygous for a loxP flanked Rac1 allele (Rac1flox/flox) with CD2-Cre mice that delete Rac1 early in T cell development. As seen in Fig. 4E, the absence of Rac1 as well as Rac2 decreases the efficiency of TCR-induced apoptosis in activated T cells from both Rac1−/− and Rac2−/− deficient mice. To study the redundancy of Rac1 and Rac2 in RICD, CD4+ T cells from compound Rac-deficient mice in which one allele of Rac1 was left intact (Rac1+/−Rac2−/−) were used. Rac1−/−Rac2−/− deficient T cells could not be obtained because of severe T cell developmental defects in Rac1−/−Rac2−/− mice (C. Dumont and V. L. J. Tybulewicz, unpublished data). Rac1+/−Rac2−/− CD4+ T cells had greater defects in RICD than Rac1−/− or Rac2−/− T cells when compared with the WT mice of the same genetic background (Fig. 4F). T cells from all of these Rac mutants underwent normal apoptosis in response to oligomerized FasL (Fasl LZ-FLAG), a strong inducer of Fas apoptosis (WT, 72.5%; Rac1−/−, 71.3%; Rac2−/−, 75.7%; Rac1+/−Rac2−/−, 64%, p = NS), indicating that Fas signaling itself is not dependent on Rac GTPases.

In this study, we have shown that Rac small GTPases are essential for sensitization of activated T cells to undergo Fas-mediated apoptosis. Lipid raft translocation of Fas, which is necessary for Fas sensitization, is also reversed by Rac1 siRNA. Rac1 promotes microfilament remodeling and repositioning of the centrosome toward the area of contact with target cells (18). Actin reorganization is dependent on the F37 residue of Rac1, indicating likely cooperation between these two Rac isoforms in mediating RICD. Although Rac2-deficient cells have been reported to have activation defects (19), under the conditions used here proliferation, viability, and activation marker expression were not impaired in either Rac1−/− or Rac2-deficient T cells (data not shown).

Our results suggest that the modulation of Rac1 though the TCR or other receptors may alter the threshold for Fas-mediated elimination of chronically stimulated T cells. We have not observed autoantibody production or autoimmune pathology in younger Rac-deficient mice, but this will need to be studied further. Signaling through the TCR or another surface molecule, CD44, which also activates Rac1, enhanced Fas-induced apoptosis. T cells from CD44-deficient mice are resistant to RICD and also had increased pathology in an autoimmune hepatitis model regulated by Fas-mediated elimination of activated T cells (20). Activation of Rac1 may be a novel way to
enhance the sensitivity of autoreactive T cells to apoptosis, a strategy that could be useful in the therapy of autoimmune diseases.

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Disclosures

The authors have no financial conflict of interest.

References